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Polymerase Chain Reaction-based Suppression of Repetitive Sequences in
Whole Chromosome Painting Probes for FISH

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Running title: Cot-depleted Probes by PCR

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Abstract

We have developed a method to suppress the PCR amplification of repetitive sequences in whole chromosome painting probes by adding Cot-1 DNA to the amplification mixture. The repetitive sequences in the Cot-1 DNA bind to their homologous sequences in the probe library, prevent the binding of primers, and interfere with extension of the probe sequences, greatly decreasing PCR efficiency selectively across these blocked regions. A second labeling reaction is then done and this product is resuspended in FISH hybridization mixture without further addition of blocking DNA. The hybridization produces little if any non-specific binding on any other chromosomes. We have been able to successfully use this procedure with both human and rat chromosome probes. This technique should be applicable in producing probes for CGH, M-FISH and SKY, as well as reducing the presence of repetitive DNA in genomic libraries.

Key words: FISH, polymerase chain reaction, genomic DNA amplification, whole chromosome probes

Introduction

Modern cytogenetic techniques, including fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), Multiplex-FISH (M-FISH) and Spectral karyotyping (SKY), are extensively used in both diagnostic and research laboratories (Fan 2002). The probes used in these techniques contain both unique and repetitive sequences, which bind to target DNA. The labeled repetitive sequences are suppressed from binding to the target by the addition of unlabeled competitive blocking DNA, either genomic or more usually Cot-1 DNA (Pinkel, Gray et al. 1986; Lichter, Cremer et al. 1988). This process requires large amounts of blocking DNA, often 20-50-fold excesses, that is expensive if Cot-1 DNA is used and purchased commercially.

Recent publications have described a method for removing repetitive sequences using affinity chromatography to produce PCR-amplifiable, chromosome-specific painting probes (Craig, Kraus et al. 1997; Durm, Schussler et al. 1998; Bolzer, Craig et al. 1999). This method solves the problem of having to use high levels of expensive Cot-1 DNA, but requires multiple PCR amplifications and affinity chromatography purifications for many of the probes. Furthermore, the initial outlay for materials can be rather expensive.

Here, we describe a simple method to produce virtually unlimited quantities of Cot-1 depleted whole chromosome-specific painting probes (WCPs). The entire process can be done in less than one day and yields probes with high specificity without the use of additional competitor DNA. Human chromosome X and rat chromosome 1 specific probes were prepared by

microdissection of normal metaphase chromosomes. The microdissected chromosomes were then amplified using the degenerate oligonucleotide-primed PCR protocol (DOP-PCR) as described by Telenius, et al (Telenius 1992). This protocol uses a single primer containing a degenerate 6-nucleotide sequence and an initial ramping step to randomly incorporate the primer into target DNA. Once incorporated, target DNA can be further amplified with this same single primer. WCPs produced in this manner are complex enough to provide continuous coverage of target chromosomes. The WCPs produced here were tested against traditional hybridization cocktails on normal human fibroblast and rat lymphocyte metaphase spreads.

Materials and Methods

Chromosome libraries

Chromosome X was kindly provided by Dr. Maria Muhlmann-Diaz, formerly of Colorado State University and was produced using traditional microdissection techniques. Rat chromosome 1 library (RNO1) was also prepared using standard microdissection techniques (reviewed in Bussey 1996). Briefly, metaphase spreads are dropped onto glass coverslips and air-dried. Chromosomes are identified under phase-contrast, transmitted light on an inverted microscope, often with the aid of G-banding. A glass needle attached to a micromanipulator is then used to scrape desired chromosomes from the coverslip, one at a time. The chromosome DNA is then transferred to a PCR tube by breaking off the tip of the needle containing the DNA in the tube. Multiple

copies of a single chromosome are usually collected in the same tube. Collection of multiple copies of the same chromosome improves complexity and coverage of the probe on target chromosomes (Christian, Garcia et al. 1999). The tubes were then centrifuged and loaded with PCR reaction solution. The PCR reaction solution contains 1.5 μ l Thermosequenase reaction buffer (USB, Cleveland, OH), 1.5 μ l 10X dNTP solution, (200 mM dATP, dTTP, dCTP, dGTP) (Roche Molecular, Indianapolis, IN), 0.6 μ l of 100 μ M DOP primer (5'-CCGACTCGAGNNNNNATGTGG-3') (Sigma-Genosys, The Woodlands, TX) (Telenius 1992), 6 U Thermosequenase polymerase (USB, Cleveland, OH) and distilled, deionized H₂O to a final volume of 15 μ l. The microdissected chromosomes were amplified using an MJResearch PT-100 thermocycler (MJResearch, Watertown, MA). The temperature-time reaction cycling profile used was as follows; 95 °C for 10 min, 8 cycles at 94 °C for 1 min, 30 °C for 5 min, and a ramp of 0.1 °C/s to 65 °C for 5 min, followed by 12 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 5 min. This was followed by 5 min at 72 °C and a hold at 4 °C until tubes were removed (Christian, Garcia et al. 1999).

Standard library amplification and labeling by PCR

A 15 μ l reaction volume was prepared using 100 ng chromosome specific library prepared as described above, 1.5 μ l Thermosequenase reaction buffer (USB, Cleveland, OH), 1.5 μ l 10X dNTP solution, (200 mM each dATP, dTTP, dCTP, dGTP) (Roche Molecular, Indianapolis, IN), 0.6 μ l of 100 μ M DOP primer, 6 U Thermosequenase polymerase (USB, Cleveland, OH) and distilled,

deionized H₂O. The libraries were amplified using an MJResearch PT-100 thermocycler (MJResearch, Watertown, MA). The temperature-time reaction cycling profile used was as follows; 95 °C for 5 min followed by 25 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min. This was followed by 5 min at 72 °C and a hold at 4 °C until tubes were removed. Products were then purified using Qiagen's Qiaquick PCR purification kit (Qiagen, Valencia, CA) and 3 µl were run on a 1.5% agarose gel against a 100 bp DNA ladder for 60 min at 100 V. Products were in the size range of 300-800 bp.

PCR products were then diluted to 100 ng/µl in 10mM Tris-HCl, pH 8.5. A labeling step was then performed in a 50 µl volume containing 1µl of diluted chromosome library from the above reaction, 5 µl Thermosequenase reaction buffer (USB, Cleveland, OH), 5 µl of 10X dNTP solution (Roche Molecular, Indianapolis, IN), 2 µl of 100 µM DOP primer, 0.5 µl AmpliTaq LD polymerase (Perkin Elmer, Foster City, CA), 2 µl of Alexa Fluor 488-dUTP (Molecular Probes, Eugene, OR) in the case of human X or 2 µl of Alexa Fluor 594-dUTP (Molecular Probes) in the case of RNO1 and distilled, deionized H₂O. The same reaction profile was used as above. This product was not further purified.

Repetitive-sequence depletion by PCR using Cot-1 DNA

The standard amplification procedure described above was used with the inclusion of 1 µg human Cot-1 DNA (Roche Applied Sciences, Indianapolis, IN) for human Chromosome X and 1 µg rat Cot-1 DNA (Applied Genetics, Melbourne, FL) in the amplification reaction. Prior to use, the Cot-1 DNA was

ethanol precipitated and resuspended at 1 $\mu\text{g}/\mu\text{l}$ in 10 mM Tris-HCl, pH 8.5, to remove EDTA, which inhibits PCR amplification.

Metaphase Chromosome Preparation

Human metaphase spreads were prepared by growing human BJ1 cells (ATCC) to confluence in DMEM/F12 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO). The cells were then subcultured at a 1:5 dilution and incubated for 30-36 hours. Mitotic cells were collected and dropped on slides as previously described (Dugan and Bedford 2003).

Rat metaphase spreads were prepared from blood cultures obtained by cardiac puncture of 8-12 week old, male Sprague-Dawley rats following the procedure in (Tucker, Breneman et al. 1997) with minor modifications. Animals were housed in LLNL Animal Care Facility. Fresh blood was washed 2x with 5 ml RPMI 1640 (Gibco, Gaithersburg, MD) media containing 10U/ml Heparin (Sigma) and incubated in RPMI-1640 supplemented with 10% FBS (Sigma), 3 $\mu\text{g}/\text{ml}$ Concanavalin A (Sigma), 100 $\mu\text{g}/\text{ml}$ Lipopolysaccharide (Sigma), 1% L-glutamine (Gibco) and Antibiotic/antimycotic (Gibco). Cultures were incubated for 62 h at 37 °C in a humidified incubator containing 5% CO₂. Colcemid (Gibco) was then added at a final concentration of 0.1 $\mu\text{g}/\text{ml}$ for 4 h. Mitotic cells were then collected and dropped on slides as previously described (Dugan and Bedford 2003)

Fluorescence in situ hybridization

A 4 μ l volume of Alexa Fluor-488 labeled library containing 200 ng/ μ l was diluted with or without 4 μ g Cot-1 DNA in a final volume of 15 μ l containing 50% formamide, 2X SSC and 10% Dextran sulfate. The probe cocktail was then denatured for 10 min. at 84°C and incubated at 37 °C for 45 – 60 min. Target slides were prepared by dehydration in an ethanol series consisting of 2 min. washes in 70%, 85% and 100% ethanol at room temperature. Slides were air-dried and denatured for 2-3 min. in 70% formamide, 30% 2xSSC @ 72°C. This was followed by a second dehydration series. The denaturations were timed so as to be completed simultaneously. The probe cocktail was then placed on the target slide and covered with a 22 X 22 mm coverslip. The coverslip was sealed with rubber cement and the slide was placed in a sealed slide box and incubated for 1-2 days at 37 °C.

After incubation, the rubber cement and coverslip were carefully removed and the slide was rinsed 2X in 50% formamide, 2X SSC at 45 °C for 5 min per rinse. Slides were then rinsed 2X in 2X SSC at 45 °C for 5 min per rinse. This was followed by rinsing 2X in room temperature 1x PN buffer for 3 min per rinse. Finally, 10 μ l of antifade solution containing 2.5 ng/ μ l DAPI counterstain was placed on the target area and covered with a coverslip.

Image capture and analysis

Slides were imaged using a Zeiss Axioskop microscope equipped with epifluorescence and standard DAPI/FITC/Texas red excitation filters and a triple

bandpass DAPI/FITC/Texas red filter set. Images were captured using a Photometrics' SenSys CCD camera (Photometrics, Tucson, AZ) and Applied Imaging's Quips image analysis software (Applied Imaging, Santa Clara, CA).

Results and Discussion

Figure 1A shows results of hybridization of the human, whole chromosome X specific painting probe library amplified under standard conditions, i.e., without the presence of Cot-1 DNA in the PCR reaction, and hybridized, also without addition of unlabelled Cot-1 DNA. As expected, the unblocked presence of labeled, repetitive sequences common to all chromosomes results in the more or less uniform painting of all chromosomes. Figure 1B shows results of hybridization of the chromosome X library amplified under standard conditions, without Cot-1 DNA in the PCR reaction, but in this case hybridized in the usual way with the addition of a 20-fold excess of unlabeled, blocking Cot-1 DNA during probe hybridization on the slide. The presence of the unlabelled Cot-1 DNA competitively blocks painting of all but the X chromosome. Figure 1C shows the results obtained following hybridization of the chromosome X library amplified with Cot-1 DNA present in the PCR reaction, but without addition of unlabeled Cot-1 DNA during the hybridization. Thus, the blocking occurred by competitive hybridization during the PCR reaction, rather than on the slide.

This method can be expanded for use in other non-human mammalian species as shown in Figure 2. A whole chromosome probe for RNO1 was prepared by microdissection, labeled and hybridized to rat metaphase spreads in the presence of rat Cot-1 DNA. As seen in Figure 2A, this produces little to no background signal and high specificity to chromosome 1. By amplifying the chromosome library in the presence of rat Cot-1 DNA, followed by labeling and hybridization without additional Cot-1 DNA, we were able to obtain similar results as the standard procedure, Figure 2B.

The addition of a 10-fold excess of Cot-1 DNA to a PCR amplification reaction involving chromosome-specific libraries blocks, or at least drastically reduces the PCR efficiency of the amplification of the highly repetitive sequences in the library while allowing for the unimpeded amplification of unique sequences. It is thought that the presence of the Cot-1 DNA in the PCR reaction binds competitively with the DOP-primer to repetitive elements during the annealing step, minimizing the amplification of these repetitive elements. As shown in Figures 1 and 2, this technique yields hybridizations of probes of equal quality to standard procedures utilizing large quantities of Cot-1 DNA. The value of this approach lies in the fact that, once made, Cot-1 free libraries remain so, and no repetitive element blocking is ever necessary in subsequent reactions.

We have presented a method for removing repetitive sequences from chromosome-specific libraries that is quick, inexpensive and produces results equaling traditional FISH methods. We have estimated that for each initial 15 μ l PCR-blocking reaction, we can produce >1000 X 10 μ l hybridizations without

additional Cot-1 DNA. At 20 μ l Cot-1/hybridization and a cost of ~\$100/500 μ l Cot-1DNA, this comes to a savings of >\$5000.

Using this technique, a large volume of a chromosome specific DNA library can be generated in a single day. This method should be applicable to any process where the need exists for high quality libraries of unique or low-copy DNA sequences.

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Figure Legends:

Figure 1. Human, whole chromosome X painting probe library labeled with Alexa-fluor 488-dUTP and hybridized to male, BJ1 fibroblast metaphase spread. 1A) Chromosome X library amplified using standard protocol, no Cot-1 DNA during PCR, and hybridized without addition of unlabeled Cot-1 DNA. 1B) Chromosome X library amplified using standard protocol, no Cot-1 DNA during PCR, and hybridized with addition of unlabeled Cot-1 DNA. 1C) Chromosome X library amplified in the presence of Cot-1 DNA during PCR, and hybridized without the addition of unlabeled Cot-1 DNA. Scale bar = 10 μm .

Figure 2. RNO1 whole chromosome painting probe library labeled with Alexa-Fluor 594-dUTP and hybridized to male, rat lymphocyte metaphase spreads. 2A) Chromosome 1 library amplified using standard protocol, no Cot-1 DNA during PCR and hybridized with addition of unlabeled Cot-1 DNA. 2B) Chromosome 1 library amplified in the presence of Cot-1 DNA during PCR, and hybridized without the addition of unlabeled Cot-1 DNA. Scale bar = 10 μm .

Figures:

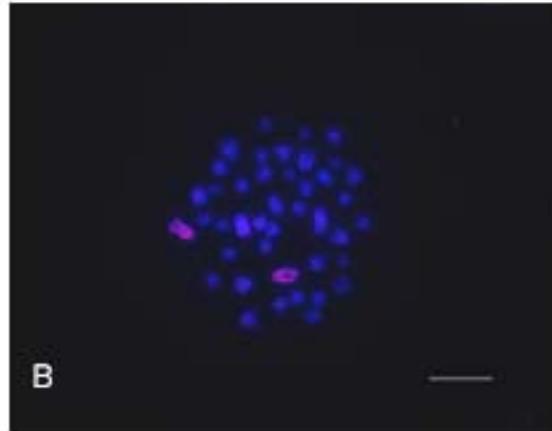
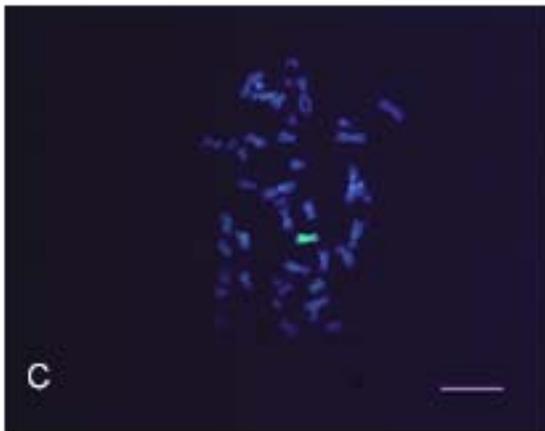
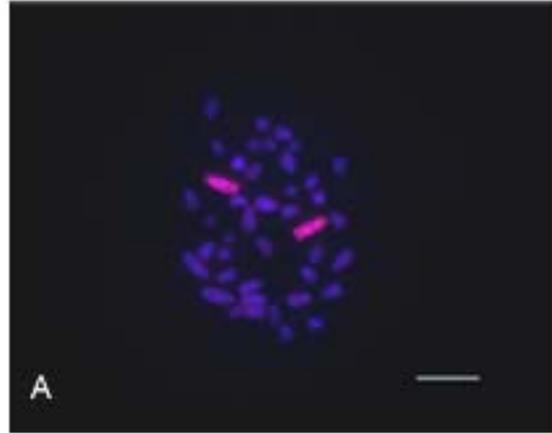
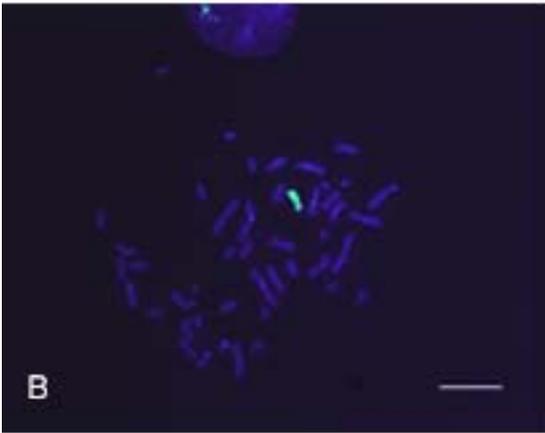
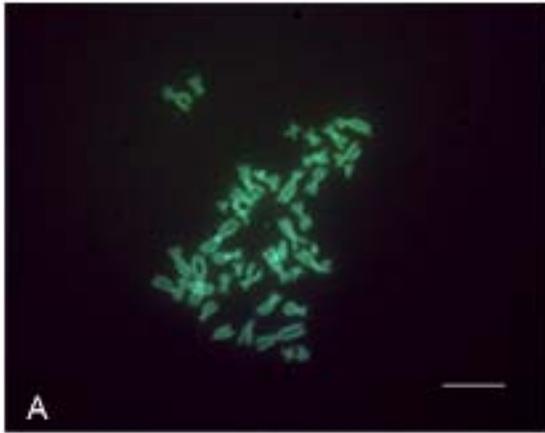


Figure 1. Human chromosome X

Figure 2. Rat chromosome 1